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A STUDY OF BACILLUS BULGARICUS.*

(*Synonyms: Bacillus of Massol, B. acidophilus, Boas-Oppler bacillus, Bacillus panis fermentati, Streptobacillus lebenis, Leptothrix buccalis.*)

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IN a preliminary note, presented at the meeting of The Society of American Bacteriologists at Baltimore, 1908, we reported the finding of a bacillus resembling *B. bulgaricus*, an organism which has been studied extensively because of the fact that Metchnikoff recommends its use in the artificial preparation of buttermilk. According to our investigations, this bacillus is the principal agent which produces lactic acid in salt-rising bread, the acid thus formed decomposing sodium bicarbonate, with the liberation of carbon dioxide. Salt-rising bread, as is well known, is started by a mixture of meal, usually cornmeal, with milk, saleratus, and a small amount of salt. The liberation of carbon dioxide causes the rising of the dough. We ascertained that the active bacillus was present in the cornmeal, since a mixture of cornmeal and sterilized milk revealed the organism in large numbers and practically in pure culture. This bacillus is somewhat difficult to cultivate, and we found that the ordinary laboratory media are quite inadequate. Sterilized milk or media prepared from milk seem the most favorable, although the addition of glucose to broth increases multiplication if the incubation temperature is high enough, i. e., 37° C. or higher.

Several descriptions of bacilli producing a large amount of acid, much more than the ordinary lactic-acid bacteria, have appeared in the literature, and the suggestion of Metchnikoff has induced several workers to take up the study again. Fermented milk beverages, especially, have been investigated, and organisms found which resemble each other closely. They are described as large bacilli, which occur singly or in filaments, produce large amounts of acid in milk (up to three per cent), and are gram positive. The descriptions are not complete, owing probably to the fact that the organisms do not

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grow readily on ordinary media; but a comparison of the reports of various authors gives the impression that the bacilli resemble each other, and may be identical. One class of these bacteria, producing large amounts of acid, is described under the collective name acidophile, a term which is misleading since the bacilli do not prefer an acid medium but are able to multiply more rapidly in the presence of a higher percentage of acid than are most other bacteria. As a matter of fact, neutral media are more suitable for the growth of these bacteria than acid media. In some textbooks we find an organism mentioned under the name *Leptothrix buccalis*. The species is not well defined and probably covers several kinds of bacteria. It occurs in saliva, forms acid in the mouth, and is considered the cause of caries of the teeth. To judge from the meager descriptions, this Leptothrix should be classed with acidophile bacteria.

Emmerling[1] saw a long bacillus in the Armenian femented milk "mazun," but he was unable to cultivate it. He also found a small bacillus forming dextro-rotatory lactic acid "which apparently is identical with Hueppe's *B. acidi lactici* and has been thoroughly studied by Günther and Thierfelder." Freudenreich[2] studied "kefir" and described similar acid-forming bacilli. Finkelstein[3] found a branching, filament-forming microorganism in the intestinal canal of bottle-fed and breast-fed infants. He isolated the organism by using broth with the addition of 0.5 per cent acetic acid and two per cent glucose. Moro[4] found a similar organism by using beerwort as a culture medium. The colonies are described as having branching edges. Rodella[5] described the colonies of a similar bacillus as resembling a ball of twine. The organism grows at high temperature only and not on ordinary media. Milk or broth with two per cent glucose is a favorable medium. From liquid media the bacilli are generally single, but sometimes long threads appear. Henneberg[6] gave account of a similar organism under the name *B. panis fermentati*. Grigoroff,[7] in Massol's laboratory, examined "yoghurt," the fermented milk of Bulgaria, and the organism he found was named *B. bulgaricus* or the bacillus of Massol. Cohendy[8] and other authors, besides studying *B. bulgaricus*, made experiments in regard to the possibility of acclimatizing it in the digestive tract of man. Bertrand and Weissweiller[9] tested the same organism as to the chemical changes brought about in milk. They conclude that the casein and butterfat are decomposed to some extent, and that the acid produced is principally lactic acid, with small amounts of volatil acids. The lactic acid is a mixture of dextro-rotatory and levo-rotatory, the former predominating. Rist and Khouri[10] made an extensive study of the Egyptian fermented milk "leben" and called the principal acid-forming organism *Streptobacillus lebenis*. Mereschewsky[11] distinguished

[1] *Centralbl. f. Bakt.*, Abt 2, 1898, 4, p 418.

[2] *Ann de l'Inst Pasteur*, 1895, 9, p. 811.

[3] *Deutsch. med. Wchnschr.*, 1900, 26, p. 263.

[4] *Wiener klin Wchnschr.*, 1900, 13, p. 114.

[5] *Centralbl. f. Bakt*, Abt. 1, 1901, 29, p 717.

[6] *Ibid* Abt. 2, 1903–4, 11, p. 168.

[7] *Rev. méd. de la Suisse Rom.*, Genève, 1905, 25, p 714.

[8] *Compt. rend. de la soc. biol.*, 1906, 60, p. 364.

[9] *Ann. de l'Inst. Pasteur*, 1906, 20, p 977

[10] *Ibid.*, 1902, 16, p. 65

[11] *Centralbl f. Bakt.*, Abt. 1, 1905, 39, p. 380.

two strains of the so-called *B. acidophilus* on the strength of finding differences in the colony-formation. The Sardinian fermented milk "gioddu" was examined by Grixoni[1] who found a bacillus which occurs in pairs or chains of six to eight members, stains at the poles or exhibits granules and does not grow on the ordinary laboratory media. Weigmann, Gruber, and Huss[2] described a bacillus which produces large amounts of lactic acid. Düggeli[3] found in "mazun" prepared from buffalo milk, sheep's milk, or goat's milk, a long bacillus which forms flocculent ("flöckige") colonies, grows well at 30° C. or more, but not at 20°. The amount of acid produced was 2.89 per cent. Piorkowski[4] examined "yoghurt" and found a large bacillus, which resembles the hay bacillus morphologically. The colonies have branches or uneven edges like a cactus plant. The optimum temperature was found to be 45° C. Sewerin[5] called attention to the identity of the Bulgarian bacillus and the *Streptobacillus lebenis* of Rist and Khouri. He studied two varieties, one forming a slimy milk, the other not; otherwise the two strains were identical. He also stated that the slime-producing variety loses this property after some time. The amount of acid produced by the slimy variety was 2.37 per cent after seven days; by the other variety 1.64. The curd forms slowly, more so than with *Strept. lacticus. B. bulgaricus* loses its vitality rapidly.

A bacillus which seems to resemble the organisms described by the authors mentioned was found by Boas and Oppler[6] in the gastric fluid of patients with carcinoma of stomach. This organism has never been studied thoroughly, chiefly because it is difficult to cultivate by ordinary methods. Morphologically it is quite similar to the acid-producing bacilli of other authors, showing granules and vacuoles in stained preparations. It occurs in filaments, and produces large amounts of lactic acid. Strauss[7] found the same bacilli in small numbers in normal gastric fluid. In stains from the stomach the bacilli have been described as staining solid with carbol fuchsin and granular with methylene blue. The granules were sometimes quite large, and the unstained portions might be mistaken for spores. The gram stain is positive. The colonies in a medium made by adding agar to gastric juice had the shape of leaves with thornlike protuberances. Milk was coagulated in one to three days. Schlesinger and Kaufmann[8] found this organism in 19 cases of carcinoma of the stomach out of 20 examined. The same authors cultivated the organism on meat-peptone agar with the addition of glucose, and in beerwort. Sternberg[9] described the colonies of the Boas-Oppler bacillus as minute points with woolly edges. The bacilli are straight or some slightly curved. They stain with the anilin dyes, and sometimes vacuoles appear, resembling spores. If the colonies are numerous, they give the plate the appearance of being covered with dust. On meat-peptone agar they grow sparingly and not below 37° C. They grow well in broth with sugars. Flagella were stained by Van Ermengen's method. Milk is coagulated after two to three days, but the property to coagulate is easily lost. Sandberg[10] gave a complete list of the literature and made a careful study of the organism. He found two types of colonies, one with woolly edges and small branches, the other more solid. The bacilli resist a high degree of acidity and are longer and larger in acid media than when the reaction is neutral.

[1] *Centralbl. f. Bakt.*, Abt. 2, 1905–6, 15, p. 750.

[2] *Ibid.*, Abt. 2, 1907, 19, p. 70.

[3] *Ibid*, Abt. 2, 1905–6, 15, p. 577.

[4] *Ibid*, Abt. 2, 1908, 21, p 95.

[5] *Ibid.*, Abt. 2, 1908, 22, p. 3.

[6] *Deutsch. med. Wchnschr.*, 1895, 21, p. 73.

[7] *Ztschr. f. klin. Med.*, 1895, 28, p. 578.

[8] *Wien. klin. Rundschau*, 1895, 9, p 225.

[9] *Wien. klin. Wchnschr.*, 1898, 11, p. 744.

[10] *Ztschr. f. klin. Med.*, 1903–4, 51, p. 80

The author claims slight motility for his organism. Rodella[1] asserted identity of the so-called acidophile bacteria, the Boas-Oppler bacillus, *B. bifidus* of Tissier, and the other bacilli forming large amounts of lactic acid. Kuntze,[2] in an exhaustive treatise, described two bacilli which differ slightly. The one shows many granules ("Körnchen Bacillus") when stained with Neisser's stain or with alkaline methylene blue, the other (*B. bulgaricus*) stains more solidly. The first bacillus grows at a lower temperature than the other. Both grow poorly on the ordinary laboratory media, but more luxuriantly on media with the addition of glucose. The colonies are like snowflakes and sometimes cloudy and dim. The author found the "körnchen" bacillus in the stomachs of calves, and believes that "yoghurt" was originally prepared by adding the stomach contents of calves to boiled milk. The filament formation is more pronounced in cultures of the "körnchen" bacillus than in those of the Bulgarian bacillus. The colonies of the former have rootlike branching; the latter are more compact. The author admits that the two organisms are probably identical. Ficker[3] found similar bacilli in smears from the esophagus of man and in human feces. With Lugol's solution and gentian violet these bacilli stain deeply blue or violet. They form filaments and stain solidly from stools, but show granules when stained from the esophagus. The author ascribed this difference in staining to the presence of starch in the esophagus and its absence from feces. The bacilli produce large amounts of lactic acid. They multiply in cases of carcinoma of the stomach because of the absence of hydrochloric acid, but in normal stomachs the presence of acid restrains their growth. They do not form spores and are not motile. They were found in nearly all of 148 cases examined, when the normal amount or even an excessive amount of acid was present. They are facultative anaerobes. They produce lactic acid in the stomach and in other parts of the digestive tract. Hastings,[4] in a short note, reported the occurrence of large bacilli producing a great amount of acid in milk soured at high temperature for several weeks.

A survey of the descriptions of the various authors quoted shows clearly that these organisms have not been studied thoroughly and under uniform and varied conditions. It is difficult, therefore, to form an opinion as to the identity of all the different forms mentioned. The differences, however, are insignificant. A few observers have described motility of the bacillus, but most have not. Otherwise the variations, such as that of colony-formation and staining properties in relation to the appearance of granules under different conditions, are not of sufficient weight to separate species. We are really dealing with bacteria of the following characteristics: They are fairly large bacilli, the length of which varies according to the composition and reaction of the culture medium. In neutral media the forms are shorter than in media containing considerable acid. The bacilli occur singly or form filaments of varying length, and often the filaments are so numerous as to appear under the microscope like a streptothrix. The form of the letter "Y" is frequently observed. The ends of the bacilli are round or square. They stain readily with the anilin dyes, and under some conditions granules appear or the poles only are stained. Young cultures are positive to the gram stain, the result being a beautiful dark blue or violet. In older cultures the gram stain is often negative; or some bacilli stain positive, others negative, so that if dilute carbolfuchsin is used as a counter stain, a part of a filament appears blue, another part red. The colonies are either solid or have woolly edges or branches. This variation is probably due, at least in part, to the composition and consistency of the culture medium. All observers agree that the colonies are very small, usually minute points. The appearance of the plates has been described as though sand or dust had been mixed with the medium. The bacilli do not grow on the ordinary laboratory media at room temperature, and sparingly at 37° C. They grow well in milk, in media prepared from milk, or if glucose is added to the medium. The most favorable reaction of the culture medium is neutral or slightly acid to phenol phthalein, but growth occurs in the presence of considerable amounts of acid, and vitality is retained in acid media when other bacteria are unable to live or multiply. The optimum temperature seems to be 40° C., some observers reporting 42 to 44°, others even 45°. Milk is coagulated, but somewhat more slowly than by the ordinary lactic-acid bacteria. Large amounts of lactic acid, up to three per cent, are produced from carbohydrates. These bacteria have been found in the esophagus, in the stomach of man and calves, in the feces of man, and in the fermented milks of the Orient.

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[1] *Centralbl. f. Bakt.*, 1908, 47, p. 445.

[2] *Ibid.*, Abt. 2, 1908, 21, p. 737.

[3] *Arch. f. Verdauungskrankheiten*, 1908, 14, p. 537.

[4] *Science*, 1908, 28, N. S., p. 656.

From these considerations it seems reasonable to suppose that one organism has been described by many authors under different names. According to the source from which it was obtained the characteristics have been found to vary somewhat, not sufficiently, however, to justify the recognition of different species. The difficulty of cultivating the organism is no doubt largely responsible for the fact that it has been rarely found, and then only when conditions were exceptionally favorable for its growth, as in fermented milks. By employing suitable media and methods of isolation we have succeeded in cultivating the bacillus from a variety of sources. The medium used was ordinary broth with the addition of 0.5 per cent glacial acetic acid and two per cent glucose. The large amount of acid restrained most other bacteria, while injuring *B. bulgaricus* but little. By these methods we succeeded in isolating cultures of the bacillus from the following material:

1. In all of 8 samples of cow feces.
2. In all of 6 samples of horse feces.
3. In all of 6 samples of human feces.
4. In 4 of 5 samples of human saliva. In 3 of these it was very numerous.
5. In 5 samples of soil where manure of cows or horses might be expected to be present.
6. In 1 out of 2 samples of soil where the presence of manure was excluded.
7. In 6 samples of market milk kept at 37° or 42° C. for several days.
8. In fodder for cattle, viz., bran, silage, and dry brewers' grains.
9. In the following foods and spices: cornmeal, sauerkraut, olive juice (from bottled olives), dill pickles, pepper mango.
10. In small numbers in 2 samples of normal gastric fluid and in exceedingly large numbers in gastric fluid from 2 cases of carcinoma of the stomach.

We did not succeed in isolating the bacillus from wheat flour, roquefort cheese, picalilli, sweet and sour pickles.

We judge from the various sources from which the bacilli were isolated that excessive acid or the absence of carbohydrates constitute unfavorable conditions for the life of the organism, and that if they gain access to substances under such conditions they do not multiply, but gradually die.

Of the seven samples of soil examined for the presence of *B. bulgaricus* we found that in earth from a paddock, where horses and cows were kept, the bacillus was present in large numbers. Soil in front of a horse stable and from the bank of a small creek contained it in fairly large numbers. In one sample obtained from the roadside where the soil had been cut recently for road improvement we found the bacillus in small numbers, and in samples from the shore of a small inland lake as well as in soil taken from beneath the roots of grass under a clump of trees we were unable to find any bacilli. In garden soil we had no trouble finding bacilli in fairly large numbers. It seems probable, therefore, that *B. bulgaricus* occurs naturally in soil, but in large numbers only when manure is present.

METHODS OF STUDY AND ISOLATION.

We found the following method successful for isolating the Bulgarian bacillus. The material to be examined was added to a tube of litmus milk and incubated at 42° C.

for 24 to 48 hours. Feces or other material containing large numbers of bacteria was added to acid broth composed of ordinary meat infusion or meat-extract broth with addition of 0.5 per cent glacial acetic acid and two per cent glucose. A transfer to litmus milk was made after 24 hours. The large amount of acid seems to restrain other bacteria from multiplying and interfering with the growth of *B. bulgaricus*. The litmus milk tubes were then used for plating in milk-serum agar, to which we also added 0.5 per cent glacial acetic acid.[1] The plates were incubated at 42° C. Microscopic colonies are usually visible in 24 hours, and are discernible to the naked eye after 48 hours. They are small and resemble colonies of streptococci, but may be distinguished from these under the hand lens by their slight irregularity of contour. Under the No. 3 objective they have the characteristic woolly appearance of *B. bulgaricus*. If present in the original material, they occur on such a plate in nearly pure culture, other forms having been suppressed by the high temperature and by the acidity of the agar. The litmus milk tubes themselves are, after 48 hours, an indication of the presence of *B. bulgaricus* in the material added to them. The color of the litmus caused by *Strept. lacticus* is a light pink, but that caused by *B. bulgaricus* soon becomes a deep vivid pink, which is quite distinctive. We were soon able to tell at a glance from a series of milk tubes which were likely to prove positive for this organism.

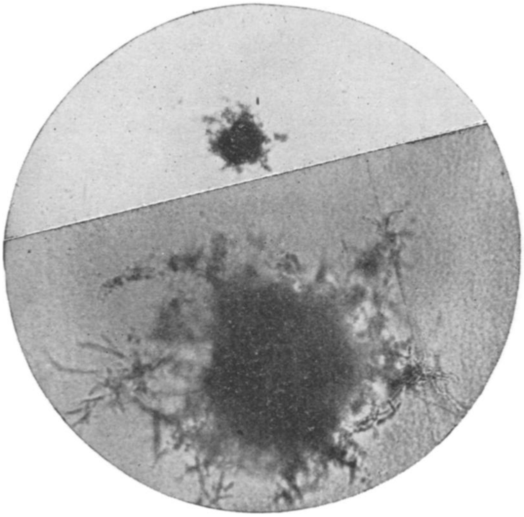

Fig. 1.—*B. bulgaricus* from normal mouth, 48-hour colonies on acid whey agar at 42° C. Small colony ×40. Large colony ×220.

Cultural characters.—On whey-agar (one per cent acid to phenolphthalein) plates at 37° to 42° C. the 24-hour colonies are minute and almost indistinguishable to the eye from streptococcus colonies. If numerous they show on the plate like fine grains of sand. Under the microscope the edges of even the smallest colonies are seen to be broken or fringed and the 48-hour colonies are characteristically densely matted, woolly at the center and branching at the edges (Fig. 1).[1] The colonies remain small and are mostly under the surface. On acetic-acid agar they appear readily and numerously, but remain minute and are smaller than streptococcus colonies. On plates from the gastric juice of a case of carcinoma two somewhat different types could be distinguished, one being the same as usual, the other more dense.

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[1] Preparation of milk-serum agar: Boiling certified milk is coagulated by addition of a few drops of acetic acid. The serum is filtered, the reaction made 1 per cent acid to phenolphthalein, 1 per cent Witte's peptone and 2 per cent glucose added, and after solution 1.5 per cent agar is dissolved in it. The whole is then clarified with the whites of eggs or dry egg albumin.

On whey-agar slants the cultures show a good development both on the surface and in stabs. The surface growth consists of a large number of small colonies, often more or less confluent so as to form a delicate veil. The stab is fairly vigorous and granular.

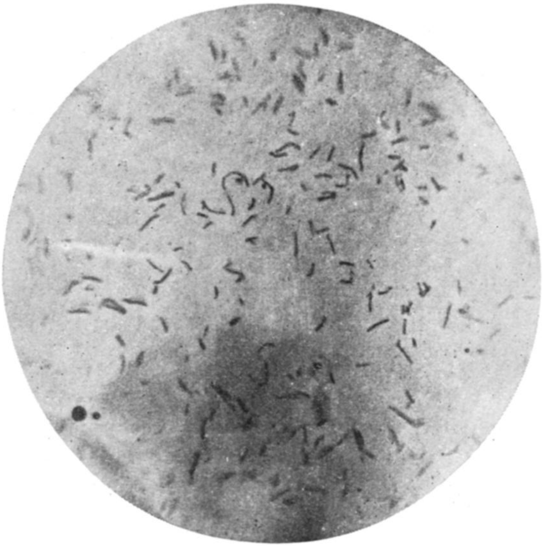

FIG. 2.—*B. bulgaricus* from milk. Pure culture on whey agar. ×300.

Milk, as mentioned above, is the most favorable medium. It is coagulated more slowly than by *Strept. lacticus*, usually not before 48 or 72 hours at 37° C., often longer. Coagulation takes place more rapidly at 42° than at 37°, and the power of acid-production is increased somewhat by reinoculation from tube to tube of milk at 42° as soon as the curd is formed, i. e., the interval shortens. The coagulum is soft, smooth, and easily broken up. Whey is rarely separated. Some strains form sliminess of the milk, but this power is readily lost. Milk cultures have a peculiar cheesy butyric-acid odor and the taste after about 10 days is extremely acid and acrid.

Ordinary laboratory media, such as plain agar, broth, potato, or gelatin, allow little or no development. Some growth occasionally takes place on glucose agar. No gas is formed on glucose media. Vitality of cultures decreases rapidly on any medium except milk. Even a whey-agar culture soon dies out.

There is little or no growth below 37° C. The optimum temperature appears to be

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[1] The photographs in this paper were made by Mr. A. C. Hicks of this laboratory to whom our acknowledgments are due.

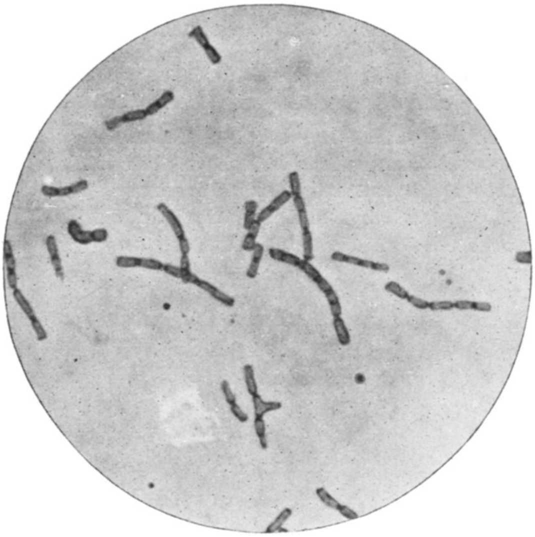

FIG. 3.—*B. bulgaricus* from milk. Pure culture on whey agar showing branched bacillus. Gram-Weigert stain. ×1,100.

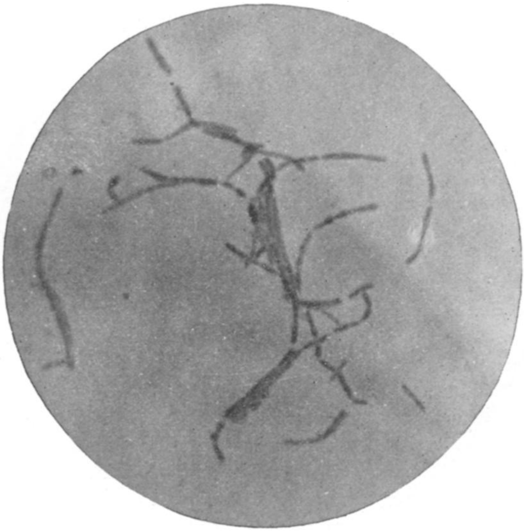

FIG. 4.—*B. bulgaricus* from normal human feces. Pure culture on whey agar. Gram-Weigert. ×1,100.

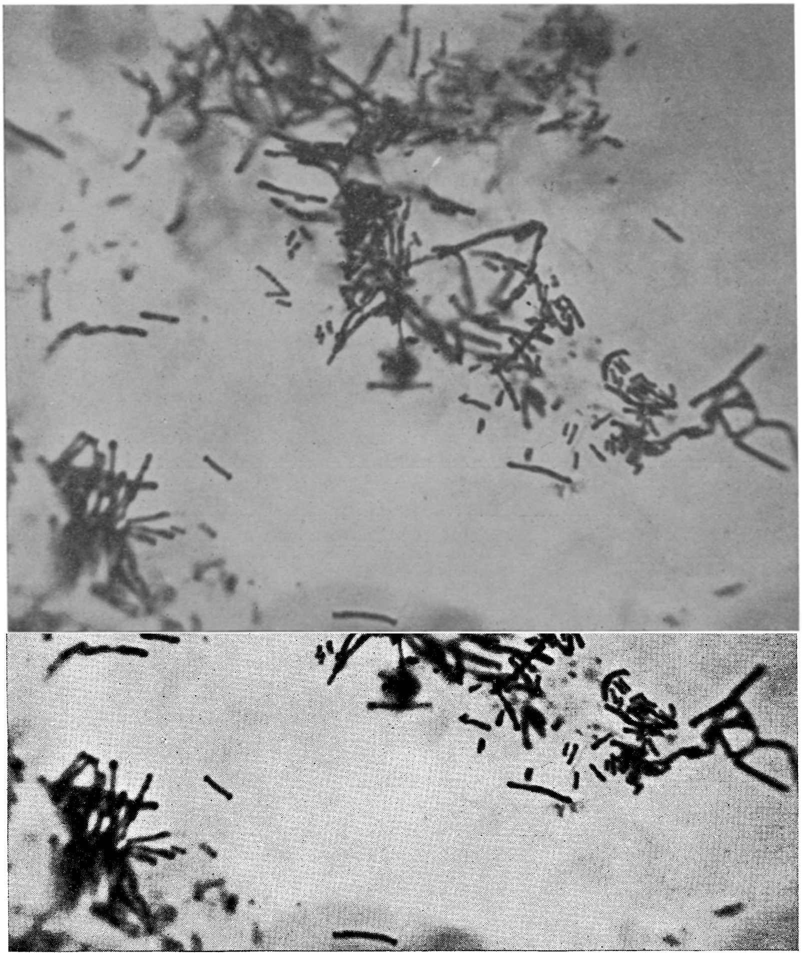

FIG. 5.—Smear from gastric fluid of a case of carcinoma of the stomach. Large and small bacilli showing polar granules. Practically pure culture. Gram-Weigert. ×1,800.

blue some strains stain solidly (streptobacilli) while others color lightly but show deeply stained round granules. The same strain may show deeply stained granules in milk and stain solidly when grown on agar. A stained preparation made from one colony shows great variation both in length and thickness of the bacilli. From milk cultures the appearance of the organism is very characteristic, consisting of long irregularly stained filaments and single bacilli, both showing unstained portions or vacuoles, and often slightly enlarged ends with unstained nodes between those of adjoining bacilli. In many cases the granules were only indicated by a somewhat darker staining at the ends of the cell. Curved and warped organisms are sometimes seen as well as tangled

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[1] Both smears and culture preparations are best stained by the Weigert modification of Gram's stain (decolorization in anilin-xylol and washing in xylol). The bacilli in a loopful of milk culture are difficult to demonstrate by other means, but show clearly, especially the granules, by this method.

masses of threads. In a smear made directly from the gastric juice of a case of carcinoma a great variety of forms could be seen. Short forms with rounded ends and beautiful granules were present, as well as long, large forms showing both distinct granules and darker staining portions. The long, solidly staining form was most prevalent, and long threads were observed. The organisms were exceedingly numerous in this preparation (Fig. 5).

PRODUCTION OF ACID IN MILK.

In order to compare the different strains isolated both with each other and with recognized strains of *B. bulgaricus* obtained from other laboratories, we examined a series carefully with respect to the power of acid-production in milk. Flasks containing 300 c.c. of sterilized milk each were inoculated by pouring into them 5 c.c. each of 48-hour-old milk cultures of the different strains. One set of flasks was also inoculated with a mixture of *B. bulgaricus* and a strain of *Strept. lacticus* of the same age and grown at 37°. The flasks were incubated at 37°, 5 c.c. being removed from each at certain intervals by means of sterile pipettes, and the acidity determined by titration at boiling temperature with $\frac{1}{20}$ normal NaOH and phenolphthalein as indicator. The amount of acid in the following table is expressed in terms of lactic acid.

TABLE I.

ACID-PRODUCTION IN MILK BY *B. bulgaricus* FROM DIFFERENT SOURCES.

SOURCE	ACIDITY IN TERMS OF LACTIC ACID AFTER								
	1 Day	2 Days	3 Days	6 Days	10 Days	17 Days	23 Days	31 Days	42 Days
Cow feces......	0.19	0.34	0.52	0.95	1.25	1.44	1.68	1.68	1.70
" "	0.22	0.62	1.28	1.93	2.48	2.73	2.72	2.73	2.73
" "	0.18	0.20	0.20	0.88	1.03	1.15	1.25	1.40	2.12
" "	0.25	0.30	0.39	0.90	1.39	1.54	1.54	1.53	1.53
" "	0.19	0.29	0.39	0.85	1.33	1.51	1.54	1.52	1.52
" "	0.17	0.20	0.22	0.35	0.56	0.68	0.85	0.89	0.93
" "	0.15	0.20	0.24	0.26	0.28	1.25	1.70	1.84	1.98
Horse "	0.17	0.29	0.40	0.81	1.01	1.27	1.40	1.52	1.52
" "	0.18	0.34	0.57	1.27	1.71	1.97	2.05	2.10	2.10
" "	0.18	0.25	0.40	0.75	0.87	0.90	0.94	1.44	1.83
" "	0.18	0.67	1.22	1.63	1.79	1.79	1.78	....	....
Human feces ...	0.22	0.40	0.58	0.82	1.21	1.30	1.36	1.36	....
" " ...	0.17	0.28	0.48	0.99	1.13	1.16	1.44	1.55	1.71
Milk	0.21	0.73	1.13	1.87	2.17	2.44	2.49	2.65	2.85
"	0.16	0.16	0.17	0.20	0.20	0.21	0.27	0.32	0.38
Cow skin	0.18	0.22	0.24	0.36	0.54	0.83	1.08	1.07	....
Brewers' grains	0.18	0.38	0.87	1.78	2.15	2.19	2.17	....	....
Bran	0.16	0.18	0.39	0.85	1.09	1.13	1.25	1.34	1.40
Cornmeal	0.16	0.19	0.26	0.54	1.23	1.45	1.75	1.90	1.90
Acid saliva.....	0.16	0.17	0.19	0.45	0.82	0.95	1.12	1.21	1.35
Nor. "	0.14	0.32	0.80	1.12	1.39	1.42	1.55	1.50	1.50
Olives	0.14	0.15	0.19	0.22	0.29	0.32	0.39	0.70	1.23
Sauerkraut.....	0.17	0.22	0.24	0.54	0.88	1.00	1.91	1.90	1.90
Dill pickles	0.14	0.65	0.77	0.82	0.85	0.84	....	....	....
Strept. lacticus..	0.29	0.53	0.66	0.73	0.73	0.73	....	....	....

The table shows clearly that the amount of acid produced by different strains varies within wide limits. We are unable at present to account satisfactorily for this variability. The highest amount was produced by a culture sent to us from the laboratory of Mr. Simeon Keith, in Boston. One strain isolated from milk came very near producing the same amount. More than two per cent were produced by strains isolated from brewers' grains, cow feces, and horse feces. The normal temperatures of cows and horses are somewhat higher than of man, and this suggests the possibility that the vitality of the Bulgarian bacillus is preserved under such temperature conditions. It is not surprising that a large amount of acid is produced from a milk strain, since milk is the most favorable medium for the growth of the Bulgarian bacillus. Dry brewers' grains possibly furnish favorable conditions for preserving the vitality, on account of the presence of considerable amounts of carbohydrates. No relation between acid-formation and slime-production was observed. In mixed cultures (*B. bulgaricus* and *Strept. lacticus*) the amounts of acid were generally smaller than in pure cultures, but the initial acid-formation was more rapid and the milk coagulated earlier (Chart I.).

The percentage of volatil acids was determined in several cultures, including the one obtained from Mr. Keith, by passing steam through 100 gm. of the sour milk and collecting the distillate in a definite amount of $\frac{1}{20}$ normal NaOH. This process was continued until no more acid passed over, and the excess of NaOH was found by titration with $\frac{1}{20}$ normal HCl. Thus we determined that the amount of volatil acid formed was 5.8 to 6.1 per cent of the total amount produced. The remaining acid was taken to be lactic acid. The optical activity of the lactic acid was determined after preparing the zinc salts in the usual manner. Solutions of these zinc salts were optically inactive and the mother liquor obtained after removing the salts was found to be inactive also. Therefore the lactic acid produced by *B. bulgaricus* is inactive without a trace of active acid.

The Bulgarian bacillus decomposed the casein and the butterfat to some extent. We have not determined the exact nature of this process, but the odor of butyric acid develops after several days, and the taste, besides being extremely sour, is somewhat bitter, perhaps due to the formation of caseones.

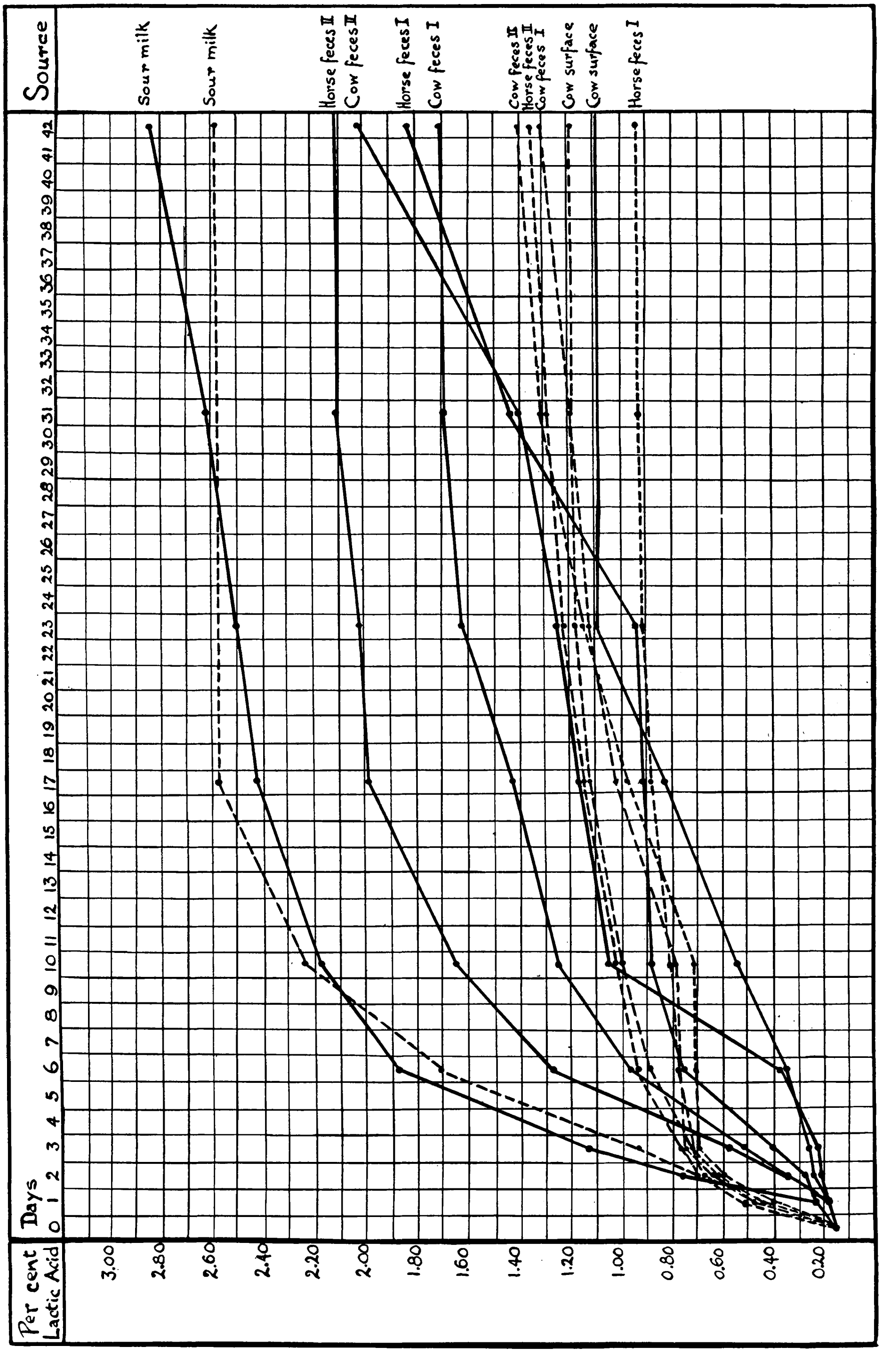

CHART 1.—Production of lactic acid in milk at 37° C. Solid line=*B. bulgaricus*; broken line=*B. bulgaricus*+*Strept. lacticus*.

The presence of *B. bulgaricus* in the apparently normal mouth, stomach, and feces of all but one case examined is a fact of considerable interest in view of the attempts which have been made to establish this organism in the intestinal tract by feeding.[1] Considering the widespread distribution which our methods of isolation have shown, there is little doubt that the lactic-acid bacillus is constantly being ingested with milk and other foods and is constantly present in the intestinal tract in some numbers, varying with the amount of normal acidity or other conditions. The number of colonies found on our plates after identical methods of enrichment and incubation differed greatly with respect to saliva from different individuals, as did also the seeming degree of vigor of the culture obtained. It is possible that the presence in the mouth of large numbers of *B. bulgaricus*, because of its lactic-acid-producing properties, may be a cause of caries of the teeth. Smears from normal stomach contents as well as from some samples of saliva often failed to show any gram-positive bacilli, but by the selective method described above we had no difficulty in final demonstration. The ingestion of milk containing large quantities of *B. bulgaricus* serves then only to increase the numbers of an organism already an ordinary habitant of the intestinal tract, and its presence after such a course of feeding is not necessarily a proof of its permanent acclimatization there in large numbers.

CONCLUSIONS.

1. *Bacillus bulgaricus* is widely distributed in nature and may be isolated by means of incubation in milk at high temperature and subsequent plating on a medium containing 0.5 per cent glacial acetic acid and two per cent glucose.

2. *B. bulgaricus* occurs normally in human feces, in the feces of cows and horses, also in a variety of sour and aromatic foods, in food for cattle, in normal saliva, in normal gastric juice and in gastric juice when hydrochloric acid is absent, in various fermented milks, in ordinary market milk, and in soil, both manured and not manured.

3. Some bacilli isolated from various fermented milks, the so-called acidophile bacilli found in the intestinal tract of man, the so-called Boas-Oppler bacillus, *B. panis fermentati*, *Streptobacillus lebenis*,

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[1] Herter and Kendall, *Jour. Biol. Chem.*, 1908, 5, p. 293; Cohendy, *Compt. rend. de la soc. biol*, 1906, 60, p. 364; Metchnikoff, *The Prolongation of Life*, New York, 1908.

an organism sometimes called *Leptothrix buccalis*, and possibly *B. bifidus* of Tissier are identical with *B. bulgaricus*.

4. *B. bulgaricus* grows poorly on the ordinary laboratory media and has therefore probably been frequently overlooked by investigators. Milk or media composed of whey, peptone, and glucose are most favorable.

5. The optimum temperature for growth is about 42° to 44° C. The Bulgarian bacillus does not grow at ordinary room temperature.

6. *B. bulgaricus* grows as well under anaerobic as under aerobic conditions.

7. The vitality of *B. bulgaricus* is readily lost, especially in competition with other bacteria under ordinary conditions.

8. The amount of acid produced in milk by *B. bulgaricus* may reach more than three per cent. The acid produced is composed approximately of six per cent volatil acid and 94 per cent lactic acid of the optically inactive variety.

9. The casein and fat in milk are partially decomposed by the activity of *B. bulgaricus*.

10. The presence of *B. bulgaricus* in normal saliva may be responsible for caries of the teeth, because of its ability to produce large amounts of acid from carbohydrates.

11. The presence of *B. bulgaricus* in human feces after ingestion of milk fermented by this organism cannot be considered positive proof of its permanent acclimatization in the digestive tract.